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HISTOCHEMICAL STUDIES ON DPN DIAPHORASE SYSTEM IN HUMAN TUMOR CELLS

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INTRODUCTION

The biochemical significance of diaphorase or coenzyme factor has been emphasized since by Adler & Euler[1-6] and Green & his coworkers.[7-12]

The histochemical studies, however, on the enzyme system have started much later. Farber and his coworkers ('54-'56)[13,14,15] have originally reported the histochemical methods for the demonstration of DPN- and TPN-diaphorases based upon the principle to utilize tetrazolium salts as a hydrogen acceptor. The author has also studied the histochemical detection of lactic dehydrogenase-DPN diaphorase system in the previous report and suggested its marked activity in tumor cells.[16]

The purpose of this paper is to present the interesting histochemical patterns of the enzyme system in gastric cancer and other neoplasms and to describe its biochemical significance for the metabolic pathway of malignant tumors.

MATERIALS AND METHOD

Most of the tissues were obtained from the surgical operation under the diagnosis of gastric cancer and pulmonary cancer, and also from the necropsy cases of various malignant tumors; 43 samples of gastric cancer, 4 pulmonary cancer, each case of liver metastasis of gastric cancer, hepatoma, breast carcinoma, embryonal carcinoma of the testicle, multiple myeloma and malignant lymphoma were included. The additional examinations of the chronic gastritis, incident to most of gastric cancers, and of benign gastric polyp were also carried out.

The fresh frozen sections of these tissues were made in a cryostat after rapid chilling of the materials by sticking on the out-side wall of a glass vessel in which dry ice-acetone or -alcohol was filled, and those were successively fixed in cold acetone about a half or an hour. The specimens 10~15 micron in thickness were incubated in the freshly prepared reaction mixture at 37°C. Since the reaction mixture required expensive chemicals, a technique employing small amounts of the mixture was utilized. The incubation was carried out in small closed glass vessels in which slide glasses sticking the specimens were horizontally supported.

Two or three tenth of one ml. of the reaction mixture preheated to 37°C was placed on the sections on each slide glass. A few amounts of water added in the vessels in order to prevent the drying of the mixture during the incubation.

The incubating mixture contained the following reagents,

0.1 %	Neotetrazolium chloride (or Nitro BT)	2 parts
0.1 M	Sod. lactate (adjusted to pH 8.5 by NaOH)	2 parts
0.4 %	DPN	2 parts
0.1 M	KCN	1 part
0.1 M	AMPD–HCl buffering solution (pH 8.5)	2 parts

Other mixture in which DPNH was substituted for lactate and DPN was also used.

The incubation period was about 10 to 15 minutes at 37°C, and was shortened when the reaction was strong. The initial color of the reactive tissues was pink, and by increasing the incubating period, progressively violet precipitate was yielded. The additional incubation resulted in the formation of blue color, but the excessive precipitation of diformazan disturbed the cytological examinations.

RESULTS

1. Chronic gastritis.

At the most cases of gastric cancer, the neighboring non-cancerous mucosa shows the histological pattern of atrophic or hypertrophic gastritis. With the lactic dehydrogenase–DPN diaphorase procedure, atrophic mucosa exhibited the decreased activity staining in pale red or pink color; the surface epithelium and thin atrophic layer of gastric glands, containing sparcely scattered remaining chief cells, stained in light color. The remainder of parietal cells, which normally showed the most intense activity and were relatively resistant to atrophic and degenerative process, still stained intensely in violet color. But the reaction was also weaker than that of normal glands. Somewhat productive interstitial connective tissue cells often showed a moderate activity.

On the other hand, at the cases of hypertrophic gastritis, the gland cells of the lengthend pits, which often showed the intestinal metaplasia containg goblet and Paneth cells, revealed the moderate reaction except for mucoidal substances. Sometimes this histochemical pattern resembled that of the relatively differentiated type of cancer.

Lymphfolicles which often formed in the deep portion of mucosa associated with chronic inflammatory process, showed light or no staining.

2. Benign adenomatous polyp.

The columnar tumor cells of well differentiated adenomatous polyp stained only in light red or pink color. However, it was merely one case and so will need further examinations.

3. Gastric cancer

The strict histological classification of gastric cancer is not easy and, as a matter of fact, most of gastric cancers are adenocarcinomas. The author has conveniently devided it in 4 types upon the basis of histological and histochemical patterns, although with some difficulties such as containing histological findings to belong partly to type 1 and partly to type 2 or 3 in the same case. This classification may be suitable not to the summarization of each case but rather to the description of respective pattern of each specimen.

The classification of the present cases are as follows;

Type 1	relatively differentiated type	8 cases
Type 2	relatively undifferentiated type	16 »
Type 3	degenerating type	10 »
Type 4	undifferentiated type	8 »

One squamous cell carcinoma of esophagus origin was also examined.

a) Relatively well differentiated adenocarcinoma (Type 1).

Well differentiated columnar or cuboid epithelial cells with round or elongated nuclei in basal portion proliferated at the mucosa with regular or irregular gland formation and infiltrated into submucosal layer beyond muscularis mucosae.

Two malignant polyps were also included in this type.

With the histochemical procedure for the eyzyme system, the cancer cells moderately stained in violet color with crude formazan sedimentation but the precipitation was not so fine as of normal parietal cells.

The invading margins of the tumor clusters more intensely stained and the superficial and central areas were rather lighter although degenerative tendency was not prominent in this type. These intracellular stainings did not work on the cytoplasmic vacuoles and nuclei.

b) Relatively undifferatiated adenocarcinoma and medullar type (Type 2).

The relatively undifferatiated type showed various histological features; secreting or nonsecreting flat epithelial cells proliferated with irregular gland formation such as cords, highly atypical glands, and almost medullar solid clumps, some of which were accompanied with stromal hyperplasia but not so marked as "Scirrhus".

With the enzyme procedure, the undifferentiated tumor cells stained in violet color with somewhat crude or fine formazan sedimentation and this staining reaction was most intense at the invading margins or infiltrating solid cells of submucosa or muscle layer. The formazan precipitated on the small cell aggregates was mostly fine and dense. On the other hand, at the central portion of medullar proliferation, the reactions were relatively weak. The cytoplasmic vacuoles containing mucus and nuclei exactly did not stain. The atypical glands sometimes showed the histochemical patterns of intense periluminal staining in contrast with weak reaction of basal portion.

The poorly differentiated cancer often illustrated the variations, for example, showing side by side partly differentiated glands and smaller rounded or polygonal cells without any gland formation. These anaplastic cells showed most intense reactions or almost negative. The latter was degenerative or necrotic tumor cells.

As the exceptional findings, 3 cases did not intensely stained.

c) Degenerative type (Type 3).

The rapidly growing tumor cells often tend to degeneration and necrosis, especially in fungating type. A typical type of degeneration is so-called colloid, mucoid or gelatinous carcinoma. The intense formation of mucus results in the floating of degenerative cancer cells in it.

In this type, the lactic dehydrogenase—DPN diaphorase procedure revealed various activities. The proliferating solid masses and nondegenerative cells stained in intense color, and otherwise, the floating degenerative cells were weak or no color with some exceptive cells to show marked activity. Generally, any cancer cell in the necrotic region did not stain. The so-called "signet ring cells" showed merely weak or no staining reaction.

d) Undifferentiated type (Type 4).

In eight cases, the tumors were composed of completely undifferentiated cells almost without any gland formation. The anaplastic cancer cells with marked anisocytosis and anisokaryosis proliferated and infiltrated in cluster even into the muscle bundles. The isolated anaplastic cells were also scattered in the loose or dense connective tissues.

Most of this type were included in so-called Carcinoma simplex. In some cases, however, atypical small glands were observed and there were signet ring cells in the scattering cell groups.

For the histochemical procedure, the anaplastic crude cells stained most intensely with dense precipitation of fine diformazan. The small anaplastic cancer cells were easily distinguished from noncancerous migrating or histiocytic cells by the marked enzyme reaction. However, signet ring cells, as already described, yielded light or no staining.

Thus, the histochemical characteristics of lactic dehydrogenase—DPN diaphorase at gastric cancers were summarized as follows; the most cancer cells had a strong activity, especially the poorly differentiated and peripherally invading cells were markedly positive, and otherwise, the degenerative and necrotic cells such as signet ring form weak or negative.

The substitution of DPNH for lactate and DPN in the reaction medium gave the identical results.

4. Other tumors.

a) Esophagus cancer.

This was a case of squamous cell carcinoma which originated from esophagus and continuously invaded into the cardiac portion.

With the enzyme reaction, the normal squamous epithelium stained intensely at the basal layer and weakened in parallel with the cell differentiation to the superficial layer.

The cancer cells which invaded into the cardiac submucosa stained in intense violet color. Although it was relatively differentiated type with keratinization and pearl formation, each layer from basal to central part uniformly stained with abrupt cease of the reaction at the keratin layer.

b) Pulmonary cancer.

Four pulmonary cancers secured at surgical operation were examined.

The first was bronchogenic papillary adenocarcinoma. The tortuous epithelium yielded the purple formazan sedimentation. The staining was completely absent at the necrotic regions.

The second was also adenocarcinoma partially of medullar type. The partially limited histological pattern resembled that of metastatic carcinoid. The tumor cell clusters also moderately stained with the enzyme procedure.

The third was typical squamous cell carcinoma with pearl formation. The histochemical pattern for the enzyme system was identical with that of esophagus cancer already described.

In additional one case (bronchogenic carcinoma), the tumor cells lightly stained with merely loose deposition of crude diformazan and homogeneous light red coloring.

c) Hepatoma.

Two hepatomas from autopsy were examined.

The aggregations of polygonal carcinoma cells resembling hepatic cells with sinusoidal stroma showed the marked activity. One case revealed more intense reaction than normal hepatic cells and the other weaker.

d) Others.

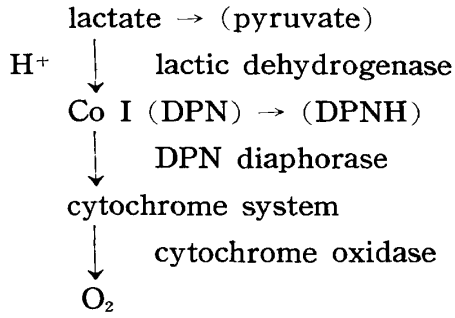
The autopsied specimens of breast cancer (mammary duct carcinoma), testicular cancer (embryonal carcinoma), multiple myeloma and malignant lymphoma were also examined. The both carcinoma cells gave the similar results to gastric cancer for the histochemical staining.

The enzyme reactions at the latter two nonepithelial tumors also yielded the diformazan formation but the intensity in each tumor cells was not uniform. Further examinations about sarcoma cells will be reported successively.

DISCUSSION

The utilization of tetrazolium salts or tellurite for hydrogen acceptor has made it possible to study a number of important oxidative enzymes in histologic setions. As a principle, the formation of water insoluble and intensely colored formazans by reduction of tetrazoles are also available for the histochemical demonstration of lactic dehydrogenase—DPN diaphorase system. Hydrogen trans-

port, as well known, at the lactate dehydration in vitro is shown as follows.



This lactate-pyruvate reaction is reversible according to their quantitative ratio.

At the histochemical procedure, however, the actual reduction of tetrazolium salts in incubation mixture was not brought about by their interaction with the dehydrogenase itself, but at the second process of reduced DPN oxidation by DPN-diaphorase and successively at cytochrome oxidation. The cytochrome oxidation may be obstructed by the addition of cyanide in the reaction mixture.

Farber et al.[13–15] have reported about the specificity of the staining reactions in their successful histochemical studies on DPN- and TPN-diaphorases. They described that the stains which had been developed, even though the reactions might be produced by the activity of single dehydrogenase systems in the tissues, were, in strict sense, specific only for DPN diaphorase and TPN diaphorase activity. At the reaction mixture of the present study, which contains lactate as substrate, it is true that the formation of formazan is strictly brought about by diaphorase activity, but the reaction may not occur without the preceding oxidation of substrate by dehydrogenase, in other words, reduction of DPN. Thus it is preferable to name "lactic dehydrogenase—DPN diaphorase system" for the present studies, and it may be rather reasonable to use reduced DPN (DPNH) itself for the "DPN diaphorase" procedure as the present additional studies and the researches by Wattenberg[17] without any other substrates such as lactate and malate.

Then, as to cyanide, the classical biochemists had considered its necessity as a keton-fixative to maintain the lactic dehydrygenase activity. But Huennekens and Green (1950)[18] have reported by their investigations of cyclophorase preparation that the tests for the dissociated apolactic oxidase were carried out at pH 8.5–9.5 with cyanide and, otherwise, for the conjugated oxidase no fixative had to be employed. The previous studies by the present author[16] have also discussed the strict necessity of cyanide for the histochemical examinations. However, the addition of cyanide, as already described, may be reasonable to restrict the reactions to the dehydrogenase and diaphorase activities inhibiting cytochrome oxidase action.

And then, as to pH of the reaction mixture, Farber et al.[13–15] have used

phosphate buffer adjusted to pH 7.4, and Wattenberg[17], 8.0. The reactions became more intense by elevation of pH, and phosphates are almost powerless as a buffering solution at the higher pH than 8.0. In the previous studies, the author has used 2-amino-2-methyl-1, 3-propandiol (AMPD)-HCl solution buffered to about pH 9.0. But, as Scarpelli and Pearse have reported[19], alkaline media (pH 10) resulted in mitochondrial swelling and enzyme diffuse. The author has used AMPD-HCl buffer solution adjusted to pH 8.3-8.5 in the present studies.

The histochemical findings of lactic dehydrogenase—DPN diaphorase and DPN diaphorase itself in gastric cancer and other tumors are summarized as follows; most cancer cells have intense activities, especially undifferentiated anaplastic and invading cells are found to be markedly positive, whereas the degenerative or necrotic cells such as signet ring cells are weak or negative. This is an interesting characteristics. Wattenberg[17] has reported the similar results in colon carcinoma.

The intense staining at malignant lymphoma cells, in spite of the weak reaction at normal lymphoid tissues, suggests the independence of the activities upon the original mother cells. Further studies about these problems will be carried out.

Generally, in malignant neoplasms, various enzyme activities are found to be diminished with some exceptions (Greenstein[20], Novikoff[21], Wattenberg[17,22], Rutenburg[23]). On the other hand, cancer cells have been repeatedly shown to possess a high glycolytic activity[24,25], in spite of the low activities of cytochromes[25,26]. This suggests the active reoxidation of DPNH during the formation of lactate from pyruvate in anerobic metabolism. In fact, the accumulation of lactic acid in tumor cells has been reported by Greenstein[20]. Moreover, the intense activity of DPN diaphorase may correspond to the anerobic metabolism for high energy source. The vigorous growth of malignant neoplasms theoretically demands immense energies. In the anerobic pathway, dehydration of intermediate 1, 3-diphosphoglyceraldehyde introduced from 3-phosphoglyceraldehyde yields 1, 3-diphosphoglyceric acid under the influence of triosephosphate dehydrogenase, and it results in DPN reduction.

The results of the present studies may be suggestive of the role of probable anerobic efficient metabolism including active DPN reduction and oxidation for a immense energy source of malignant tumors.

SUMMARY

The histochemical procedures of lactic dehydrogenase—DPN diaphorase and of DPN diaphorase itself were employed in the tissues of gastric cancer and related gastric changes. Other various neoplasms such as pulmonary cancer, hepatoma etc. were also examined.

As a characteristic reaction, most of the gastric cancer cells showed distinctive

activity by formazan formation. This staining pattern was not uniformly present in all components of the neoplasms. The enzyme activities were most intense in the cells at the invading clusters and isolated cell groups. Generally, the undifferentiated anaplastic cells stained in deep violet color. Whereas, the degenerative cells always decreased or diminished the staining reaction.

The distinctive reaction pattern in other various tumors was also suggested to be identical with gastric cancer.

The significance of these findings for metabolic pathway of tumor cells was discussed.

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Explanation of the plates

Fig. 1. Atrophic gastritis. Parietal cells remaining in the atrophic thin mucosa still intesely stained. ×100

Fig. 2. Metaplasic gastritis. The metaplasic intestinal mucosa of the pylorus portion moderately stained. Intracellular mucus did not stained. ×100

Fig. 3. Gastric polyp. The well differentiated columnar cells lightly stained. ×100

Fig. 4. Gastric cancer. Relatively differentiated cancer cells with gland formation intensely stained. ×100

Fig. 5. Gastric cancer. The reaction in the irregular glands are not uniform. ×100

Fig. 6. Gastric cancer. The invading adencarcinoma cells proved the intense staining reaction. The dilated glands of right lower portion showed the marked periluminal localization of the activity. ×100

Fig. 7. Gastric cancer. Relatively undifferentiated type. ×100

Fig. 8. Gastric cancer. The relative undifferentiated cells with irregular gland formation intensely stained. ×100

Fig. 9. Gastric cancer. Degenerating adenocarcinoma. The staining reaction proved to be intense or negative. ×100

Fig. 10. Gastric cancer. Gelatinous carcinoma. The floating cancer cells showed the various enzymatic activities. ×100

Fig. 11. Gastric cancer. The anaplastic cell clusters invading through muscle bundles stained most intensely. The muscle bundles also somewhat stained. ×100

Fig. 12. Squamous cell carcinoma which originated from esophagus mucosa and invaded into cardiac submucosa. The cancer cells markedly stained but the keratinized central portions did not stained. ×100

Fig. 13. Hepatoma. The tumor cells showed marked activity. ×100

Fig. 14. Pulmonary cancer. Papillary adencarcinoma. The tortuous carcinoma tissues stained intensely. ×100

Fig. 15. Pulmonary cancer. The marked peripheral staining is to be noticed. ×100

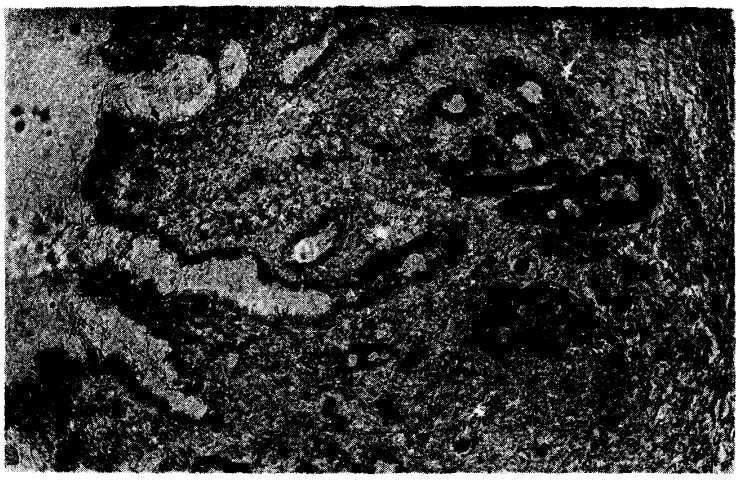

Fig. 1

Fig. 2

Fig. 3

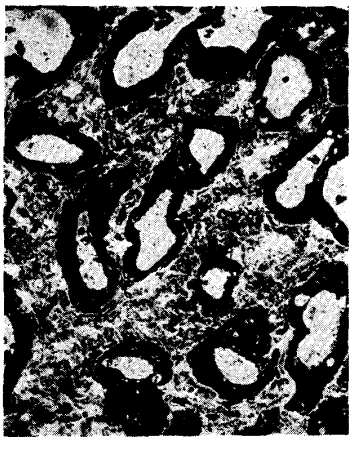

Fig. 4

Fig. 5

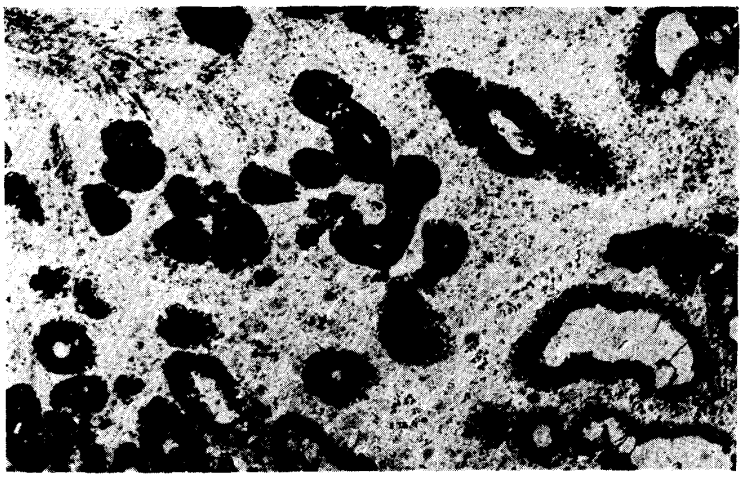

Fig. 6

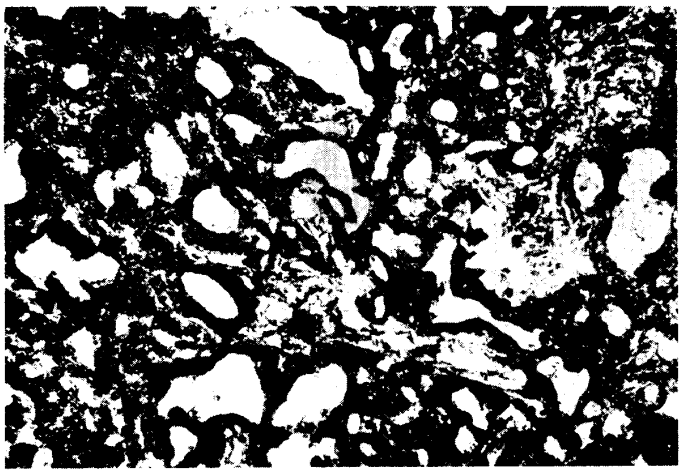

Fig. 7

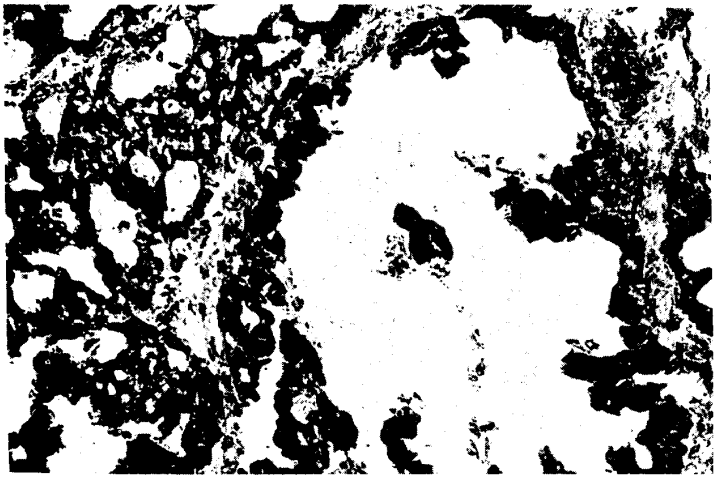

Fig. 8

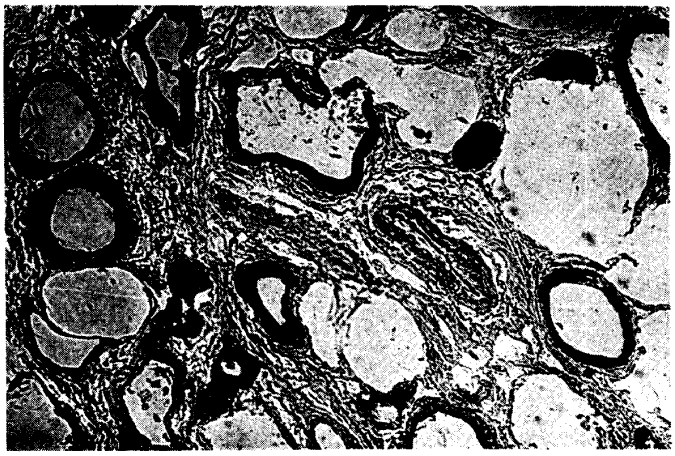

Fig. 9

Fig. 10

Fig. 11

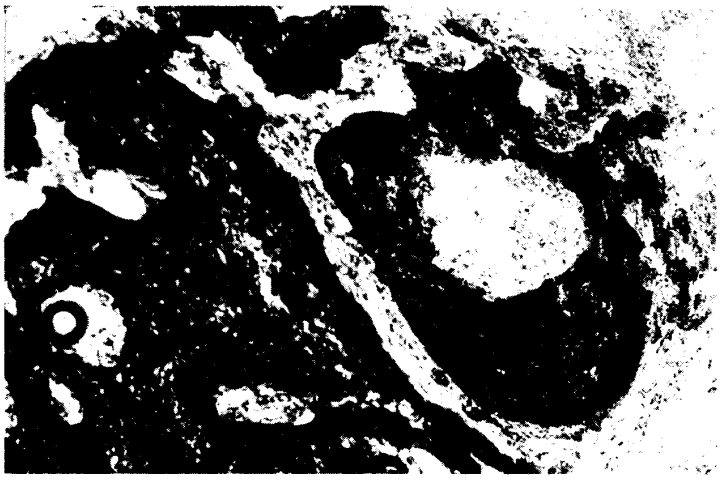

Fig. 12

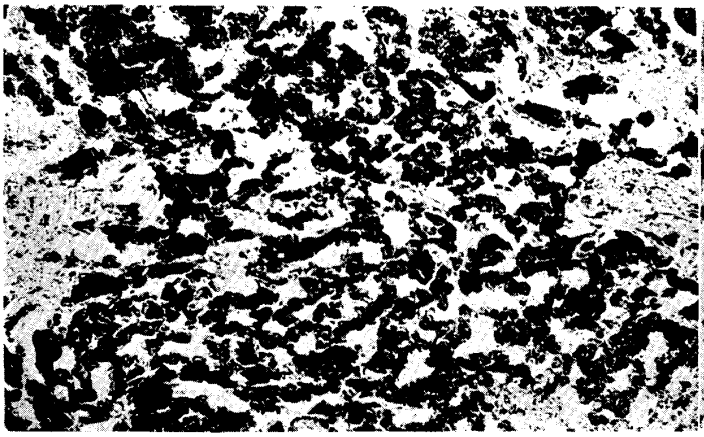

Fig. 13

Fig. 14

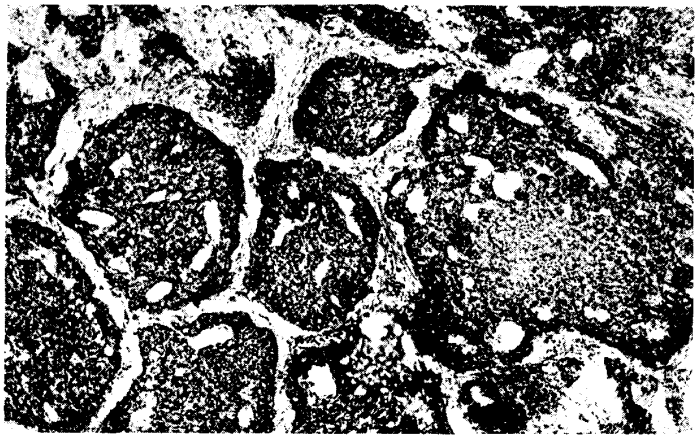

Fig. 15